

# Autophagy Inhibitor 3-Methyladenine could not Modulate Rotenone Neurotoxicity in Primary Mesencephalic Cell Culture

El Inhibidor de la Autofagia 3-Metiladenina no logra Modular la Neurotoxicidad de la Rotenona en el Cultivo Primario de Células Mesencefálicas

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**RADAD, K.; AL-SHRAIM, M.; AL-EMAM, A.; RAUSCH, W. D. & MOLDZIO, R.** Autophagy inhibitor 3-methyladenine could not modulate rotenone neurotoxicity in primary mesencephalic cell culture. *Int. J. Morphol.*, 38(3):530-535, 2020.

**SUMMARY:** Dysregulated autophagy, whether excessive or downregulated, has been thought to be associated with neurodegenerative disorders including Parkinson's disease. Accordingly, the present study was carried out to investigate whether 3-methyladenine, an autophagy inhibitor, can modulate the effects of rotenone on dopaminergic neurons in primary mesencephalic cell culture. Cultures were prepared from embryonic mouse mesencephala at gestation day 14. Four groups of cultures were treated on the 10th DIV for 48 h as follows: the first was kept as an untreated control, the second was treated with 3-methyladenine alone (1, 10, 100, 200 nM), the third was treated with 20 nM rotenone and the fourth was co-treated with 20 nM rotenone and 3-methyladenine (1, 10, 100, 200 nM). On the 12th DIV, cultured cells were stained immunohistochemically against tyrosine hydroxylase and culture media were used to measure the levels of lactate dehydrogenase. 3-methyladenine had no effects on both the survival of dopaminergic neurons and the release of lactate dehydrogenase. Rotenone significantly decreased the number of dopaminergic neurons and increased the levels of lactate dehydrogenase in the culture media. When cultures concomitantly treated with 3-methyladenine and rotenone, 3-methyladenine had no effect against rotenone-induced dopaminergic cell damage and lactate dehydrogenase release into the culture medium. In conclusion, the autophagy inhibitor 3-methyladenine could not modulate rotenone-induced dopaminergic cell damage in primary mesencephalic cell culture.

**KEY WORDS:** Autophagy; Dopaminergic neurons; Parkinson's disease; Rotenone; 3-methyladenine.

## INTRODUCTION

Parkinson's disease (PD) is a common and progressive neurodegenerative disorder affecting about 10 million people worldwide (Ball *et al.*, 2019). The prevalence of the disease is predicted to increase as the elderly people have significantly increased in the recent years (Heemels, 2016; Renaud & Martinoli, 2019). The disease results primarily from the loss of dopamine-producing neurons (dopaminergic) in the substantia nigra pars compacta (SNpc) (Khalifeh *et al.*, 2019; Yao *et al.*, 2019) and subsequent deficiency of striatal dopamine (Poewe *et al.*, 2017). Parkinson's disease as a motor disorder is characterized by a number of classic motor signs including rest tremors, bradykinesia, rigidity and stooping posture (Beitz, 2014). Non-motor symptoms such as sleep disorders, cognitive/neurobehavioral abnormalities, autonomic dysfunction, and sensory abnormalities such as anosmia, paraesthesia and pain are considered (Jankovic, 2008).

However the exact etiology of PD is still unclear, two forms of the disease exist; familial and sporadic. Familial form accounts for ~ 10 – 15 % of all PD cases and is associated with seven causal genes including alpha-synuclein (SNCA), leucine-rich repeat Kinase 2 (LRRK2), glucocerebrosidase (GBA), vacuolar protein sorting-associated protein 35 (VPS35), parkin RBR E3 ubiquitin protein ligase (PARK2), phosphatase and tensing homolog-induced Kinase 1 (PINK1), and Parkinson protein 7 (PARK7) (Verstraeten *et al.*, 2015; Kalinderi *et al.*, 2016). Sporadic PD affects ~ 85 – 90 % of the cases and is believed to return to environmental exposures, most notably, chemicals, pesticides and heavy metals (Castillo *et al.*, 2017; Pouchieu *et al.*, 2018).

Autophagy is a crucial catabolic process that keeps homeostasis inside cells and helps them to face stressors

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through delivering misfolded proteins and damaged organelles to lysosomes for degradation (Cerri & Blandini, 2018; Kirkin, 2019). Recently, increasing evidence from PD patients and PD experimental models has showed that autophagy plays an essential role in PD pathogenesis (Zhang *et al.*, 2012). In this context, Dehay *et al.* (2010) found that post-mortem PD brain samples have an increased number of undegraded autophagosomes and a lower number of autophagolysosomes. Park *et al.* (2014) observed that treatment of mice with MPP<sup>+</sup> inhibited formation of autophagosome and increased expression of  $\alpha$ -synuclein. Parganlija *et al.* (2014) showed that knock down of PINK1 in SH-SY5Y cells resulted in down-regulation of key autophagic genes such as Beclin, LC3 and LAMP-2.

Accordingly, the current study was designed to investigate the role of the autophagy-inhibitor 3-methyladenine against rotenone-induced dopaminergic cell death in the primary mesencephalic cell culture.

## MATERIAL AND METHOD

### Preparation of primary mesencephalic cell culture.

Pregnant OF1/SPF mice were cared and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. Cultures were prepared from OF1/SPF embryos on the 14th day of gestation according to Radad *et al.* (2018). Briefly, brains were released from skulls, and mesencephala were dissected and cut into small pieces in a drop of D-PBS (Invitrogen, Germany). The tissues were transferred into a Griener centrifuge tube containing 2 ml of 0.2 % trypsin solution (Invitrogen, Germany) and 2 ml of 0.02 % DNase I solution (Roche, Germany). After incubation in water bath at 37°C for 7 min, 2 ml of trypsin inhibitor (0.125mg/ml) (Invitrogen, Germany) were added, the tissue was centrifuged at 100x g for 4 min and the supernatant was aspirated. The tissue pellet was then mechanically triturated with a fire-polished Pasteur pipette. Dissociated cells were seeded at a density of 257.000 cells/cm<sup>2</sup> in DMEM (Sigma, Germany) supplemented with 4 mM glutamine, 10 mM HEPES buffer, 30 mM glucose, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 10 % heat-inactivated fetal calf serum (Sigma, Germany). The medium was exchanged with the same medium on the 1st day in vitro (DIV) and on the 3<sup>rd</sup> DIV. On the 5<sup>th</sup> DIV, half of the medium was replaced by serum-free DMEM containing 0.02 ml of B-27/ml (Invitrogen, Germany). Serum-free supplemented DMEM was used from the 6th DIV and subsequently replaced every 2nd day.

Treatment of cultures with 3-MA. A stock solution of 10 mM was prepared by dissolving 1.49 mg of 3-MA (Sigma-Aldrich, Germany) in 1 ml distilled water. Final concentrations were prepared in DMEM. On the 10th DIV, cultures were treated with 3-MA alone (1, 10, 100, 200 mM) for 48 h to investigate its effect on the survival of dopaminergic neurons and the release of lactate dehydrogenase (LDH) in the culture media.

**Treatment of cultures with rotenone.** A rotenone (Sigma-Aldrich, Germany) stock solution (10 mM) was prepared by dissolving 3.94 mg of rotenone in 1 ml DMSO. Final concentrations of rotenone were prepared in DMEM. Fresh rotenone solutions were used in each treatment to avoid its breakdown by storage. On the 10th DIV, cultures were treated with 20 nM of rotenone for 48 h.

**Treatment of cultures with rotenone and 3-MA.** Cultures were concomitantly treated with rotenone (20 nM) and 3-MA (1, 10, 100, 200 mM) on the 10th DIV for 48 h to investigate the effect of autophagy inhibitor 3-MA against rotenone-induced dopaminergic cell damage.

**Identification of dopaminergic neurons.** Dopaminergic neurons were identified by tyrosine hydroxylase immunostaining. On the 12<sup>th</sup> DIV, cultured cells were rinsed carefully with phosphate buffered saline (PBS, pH 7.2) and fixed with histochoice for 15 min at room temperature. After permeabilization with 0.4 % Triton X-100 for 30 min at room temperature, non-specific binding sites were blocked with 5 % horse serum (Vectastain ABC Elite kite) for 90 min at room temperature. Then, cells were incubated with anti-TH primary antibody over night at 4 °C and biotinylated secondary antibody (Vectastain) and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Dopaminergic neurons were counted with a Nikon inverted microscope in 10 randomly selected fields/well at magnification 10<sup>7</sup>.

**Measurement of LDH activity.** Measuring LDH activity in the culture media is usually used to quantify cell damage. At the end of each treatment, culture media were collected and used to measure LDH activity with a cytotoxic detection kit according to the manufacturer's instructions. In brief, NADH + H<sup>+</sup> produced from NAD<sup>+</sup> by LDH is transferred by diaphorase to the yellow tetrazolium salt 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) resulting in red formazan formation. The latter was measured spectrophotometrically at 490 nm with a reference at 688 nm. Supplemented medium was used as a blank and subtracted as background.

**Statistics.** Data were obtained from three experimental repeats (4 wells/experiment) for each treatment condition and presented as mean  $\pm$  standard error of mean (SEM). Comparisons between groups were made using ANOVA and post-hoc Duncan's test using IBM SPSS statistics 22. ( $p < 0.05$  was considered as statistically significant).

## RESULTS

### Effect of 3-MA on primary mesencephalic cell culture.

Treatment of primary mesencephalic cell cultures with different concentrations of 3-MA (1, 10, 100, 200 mM) on the 10th DIV for 48 h did not affect the survival of dopaminergic neurons (Fig. 1A). 3-MA also had no effect on the morphology of dopaminergic neurons. Dopaminergic neurons in both untreated controls and 3-MA-treated cultures were many with long and branched neuritis (Fig. 1B). Moreover, 3-MA did not affect the release of LDH into the culture medium compared to untreated controls (Fig. 2).

**Effect of concomitant treatment with 3-MA and rotenone on primary mesencephalic cell cultures.** Administration of rotenone (20 nM) to primary mesencephalic cell cultures

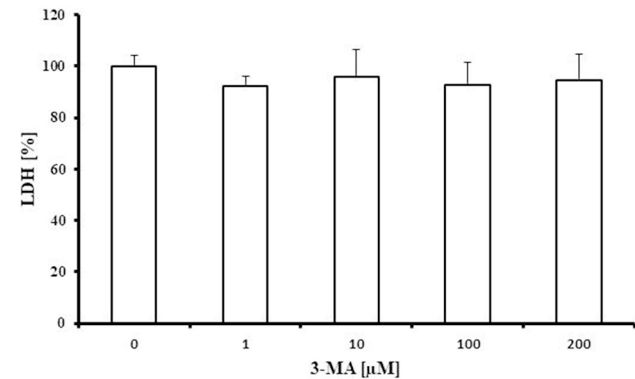


Fig. 2. Measurement of LDH in the culture medium following treatment of cultures with different concentrations of 3-MA (1, 10, 100, 200 mM) on the 10th DIV for 48 h. 100 % corresponds to the amount of LDH in the culture medium on the 12th DIV. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment ( $p = 0.548$ ).

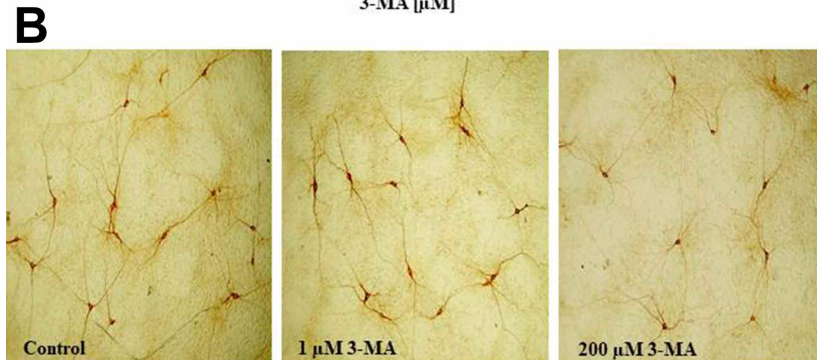
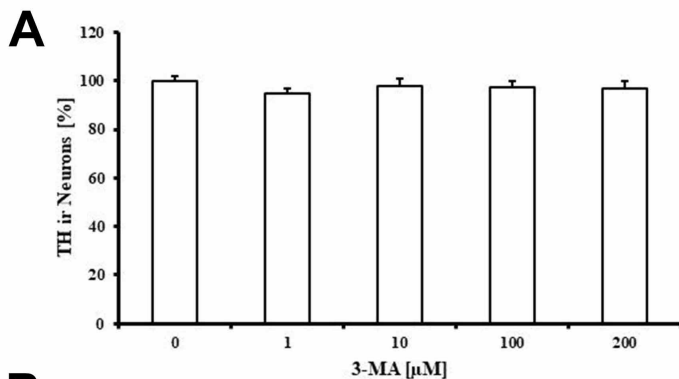


Fig. 1. Treatment of cultures with different concentrations of 3-MA (1, 10, 100, 200 mM) on the 10th DIV for 48 h. 100 % corresponds to the total number of dopaminergic neurons (the average is 27.43 cells/field) after 12 DIV in untreated control cultures. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment ( $p = 0.223$ ).

on the 10th DIV for 48 h significantly decreased the number of dopaminergic neurons by about 25 % compared to untreated controls (Fig. 3A). When cultures were concomitantly treated with 3-MA and rotenone on the 10th

DIV for 48 h, 3-MA did not modulate rotenone's effects on dopaminergic neurons compared to rotenone-treated cultures (Fig. 3A). Treatment of cultures with rotenone markedly injured dopaminergic neurons. Survived neurons appeared few with shortened and thickened neuritis (Fig. 3B). 3-MA also did not affect the morphology of survived dopaminergic neurons in rotenone-treated cultures (Fig. 3B). Also, rotenone was found to increase the release of LDH into the culture medium compared to untreated control cultures and 3-MA had no significant effect on LDH levels compared to rotenone-treated cultures (Fig. 4).

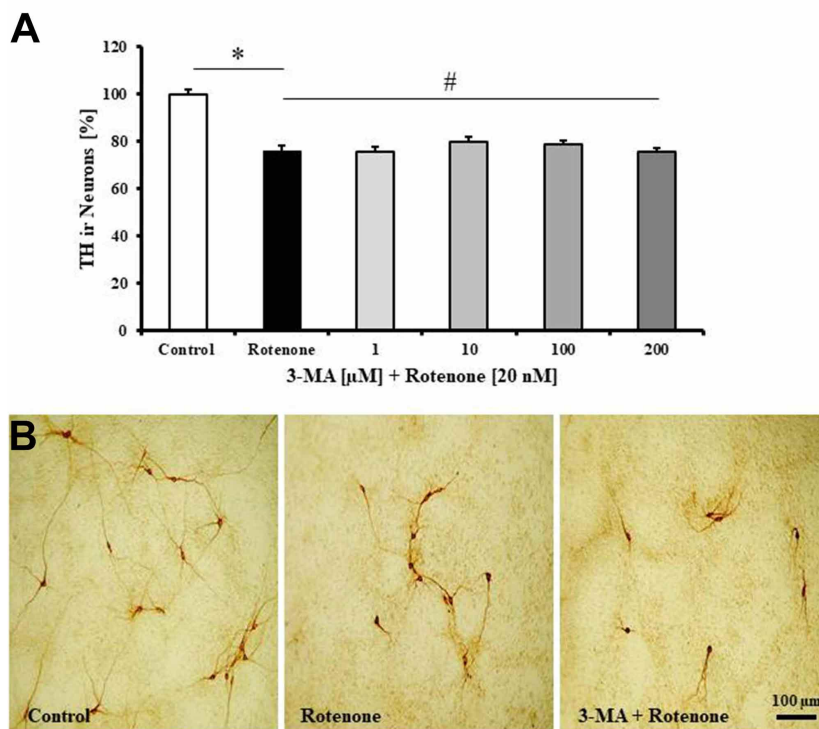


Fig. 3. Effect of concomitant treatment of cultures with 3-MA (1, 10, 100, 200 mM) and rotenone (20 nM) on the 10th DIV for 48 h. A) Number of dopaminergic neurons. 100 % corresponds to the total number of dopaminergic neurons (the average is 29.67 cells/field) after 12 DIV in untreated control cultures. Values represent the mean±SEM of three independent experiments with four wells in each treatment. (\* $p < 0.001$ , # $p < 0.184$ ). B) Representative micrographs of dopaminergic neurons after 12 DIV. Untreated control culture shows many dopaminergic neurons with long and branched processes. Rotenone-treated culture shows lower number of dopaminergic neurons with few, shortened and thickened neurites. Concomitant treatment of cultures with rotenone and 3-MA did not alter the morphology of surviving dopaminergic neurons compared to rotenone-treated cultures.

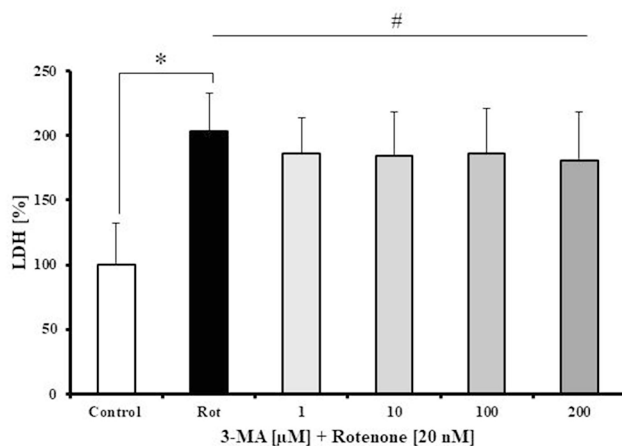


Fig. 4. Measurement of LDH in the culture medium following concomitant treatment of cultures with different concentrations of 3-MA (1, 10, 100, 200 mM) and rotenone (20 nM) on the 10th DIV for 48 h. 100 % corresponds to the amount of LDH in the culture medium on the 12th DIV. Values represent the mean±SEM of three independent experiments with four wells in each treatment (\* $p < 0.001$ , # $p = 0.064$ ).

Accordingly, the present study aimed to investigate whether inhibition of the autophagy process would modulate rotenone-induced dopaminergic cell damage in primary mesencephalic cell culture. We have previously showed that activation of autophagy by rapamycin protected dopaminergic neurons against rotenone-induced damage in primary mesencephalic cell culture.

Treatment of cultures with different concentrations of 3-MA (1, 10, 100, 200 mM) was shown to have no effect on both the survival of dopaminergic neurons and the release of LDH into the culture medium. This might occur because autophagic process is minimal and its inhibition was not effective in this well-enriched and short-lived cell culture type. In parallel, fluorescence staining of untreated primary mesencephalic cell culture with LysoTracker<sup>®</sup> deep red, a dye that retained in acidic subcellular organelles such as phagolysosomes, showed very low red fluorescence intensity (Radad *et al.*, 2015).

Administration of 20 nM rotenone to the cultures significantly decreased the number of dopaminergic neurons and increased the release of LDH into the culture medium compared to untreated control cultures. Similarly, rotenone's neurotoxicity was shown in different *in vitro* and *in vivo* models since 2000 when Betarbet and her colleagues described rotenone as a PD neurotoxin (Betarbet *et al.*, 2000). For example, Radad *et al.* (2015) showed that 20 nM

## DISCUSSION

Dysregulated autophagy has been observed in the brains of PD patients, and animal and cellular models of PD indicating the distinct role of autophagy in the pathogenesis of the disease (Lynch-Day *et al.*, 2012; Janda *et al.*, 2012).

rotenone significantly destroyed dopaminergic neurons and increased LDH release in the culture medium. Im *et al.* (2013), found that rotenone decreased the viability of PC12 cells. Tapias *et al.* (2014), observed that rotenone led to loss of dopaminergic neurons and nigrostriatal terminals in rats. This damaging effect of rotenone on cultured cells was associated with increased autophagic process as indicated by the intense red fluorescence of the LysotrackerÒ deep red compared to untreated control cultures (see Radad *et al.*, 2015). Interestingly, concomitant treatment of cultures with rotenone and 3-MA did not modulate rotenone's effects on both the survival of dopaminergic neurons and the release of LDH into the culture medium. However, inhibition of autophagy was reported to deleteriously affect aged and toxin-sensitized and tumor cells. For instance, Stroikin *et al.* (2004) found that treatment of growth-arrested human fibroblasts, a classical model of cellular aging, with 3-MA resulted in moderate increase in mitochondria with lowered membrane potential. Pickard *et al.* (2015) showed that 3-MA increased autophagic vesicle formation and apoptotic cell death of LNCaP prostate cancer cells. This toxic effect of 3-MA was reported to be due to blocking of autophagosome formation via the inhibition of class III PI3K with subsequent accumulation of misfolded proteins and altered cell organelles leading to cell damage (Wu *et al.*, 2010).

In conclusion, 3-MA, an autophagy inhibitor, does not modulate rotenone-induced dopaminergic cell damage in primary mesencephalic cell culture.

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**RADAD, K.; AL-SHRAIM, M.; AL-EMAM, A.; RAUSCH, W. D. & MOLDZIO, R.** El inhibidor de la autofagia 3-metiladenina no logró modular la neurotoxicidad de la rotenona en el cultivo primario de células mesencefálicas. *Int. J. Morphol.*, 38(3):530-535, 2020.

**RESUMEN:** Se estima que la autofagia desregulada, ya sea excesiva o con baja regulación, está asociada con trastornos neurodegenerativos, incluyendo la enfermedad de Parkinson. En consecuencia, se realizó este estudio para investigar si la 3-metiladenina, un inhibidor de la autofagia, puede modular los efectos de la rotenona en las neuronas dopaminérgicas en el cultivo primario de células mesencefálicas. Los cultivos se prepararon a

partir de mesencéfalo de ratón embrionario el día 14 de gestación. Cuatro grupos de cultivos se trataron en el 10º DIV durante 48 h de la siguiente manera: el primer grupo se mantuvo como un control no tratado, el segundo se trató con 3-metiladenina sola (1, 10, 100, 200 mM), el tercer grupo se trató con rotenona 20 nM y el cuarto se trató conjuntamente con rotenona 20 nM y 3-metiladenina (1, 10, 100, 200 mM). En el 12º DIV; las células cultivadas fueron tratadas mediante tinción inmunohistoquímica en tirosina hidroxilasa y se usaron medios de cultivo para medir los niveles de lactato deshidrogenasa. La 3-metiladenina no tuvo efectos tanto en la supervivencia de las neuronas dopaminérgicas como en la liberación de lactato deshidrogenasa. La rotenona disminuyó significativamente el número de neuronas dopaminérgicas y se observó un aumento de los niveles de lactato deshidrogenasa en los medios de cultivo. Cuando los cultivos tratados conjuntamente con 3-metiladenina y rotenona, la 3-metiladenina no tuvo efecto contra el daño celular dopaminérgico inducido por la rotenona y la liberación de lactato deshidrogenasa en el medio de cultivo. En conclusión, el inhibidor de la autofagia 3-metiladenina no moduló el daño celular dopaminérgico inducido por la rotenona en el cultivo celular mesencefálico primario.

**PALABRAS CLAVE:** Autofagia; Neuronas dopaminérgicas; Enfermedad de Parkinson; Rotenona; 3-metiladenina.

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